Volume 283, number 1, 104–108 FEBS 09759 May 1991

ADONIS 001457939100468C

The α subunit of human chorionic gonadotropin hormone synthesized in insect cells using a baculovirus vector is biologically active

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Received 5 November 1990; revised version received 3 April 1991

A recombinant baculovirus, vAc*αhCG*, having a replacement of the viral polyhedrin gene with the cDNA encoding the α subunit of hCG was used to express αhCG, an extensively glycosylated hormone, in insect cells. Virus-infected cells, 72 h pi, secreted ~ 11.3 μg αhCG/2 × 10^6 cells/ml which was identical to the native hormonal peptide in terms of electrophoretic mobility, immunoreactivity and bioactivity on association with β subunit, as evident by its binding to rat testicular cells and induction of steroidogenesis in a mouse Leydig cell bioassay system. The αhCG secreted into the medium represented ~ 20–30% of the total hCG synthesized by vAc*αCG* infected insect cells. The implications of using a very late promoter, in a baculovirus expression system, for directing the transcription of a gene whose gene product requires extensive post-translational modifications are discussed.

Birth control vaccine; Immunoreactivity; Late promoter; Post-translational modification; Secretion; *Spodoptera frugiperda*

1. INTRODUCTION

Human chorionic gonadotropin (hCG) is a product of the trophoblast. With the exception of trophoblastic and some other cancers where oncofetal genes are expressed, the rise in hCG in blood is indicative of pregnancy. hCG is being extensively used for induction of ovulation in women and for treatment of male infertility. One of the demonstrated roles of hCG is the rescue of the corpus luteum leading to the continued secretion of progesterone, which in turn sustains the endometrium and prevents its menstrual shedding. The critical role of hCG in maintaining early pregnancy is confirmed by the ability of anti-hCG antibodies, induced by active [1] or passive immunization [2], to terminate pregnancy in primates. Birth control vaccines based on the β-subunit of hCG are currently under clinical trials [3]. hCG belongs to a group of glycoprotein hormones consisting of two non-identical subunits designated α and β, which are held together by non-covalent bonds and are coded by two separate genes [4–6]. A functional hCG dimer was synthesized in monkey cells when their cDNAs were inserted in expression vectors under the control of metallothionine-1 promoter [7,8] or SV40 early promoter [9].

Conventionally purified native hCG is dissociated to obtain the subunit protein. This, however, entails recovery losses and is invariably contaminated with trace amounts of the other subunit. One of the objectives of the present work was to express the α subunit of hCG independently by recombinant DNA technology. The baculovirus expression vector system utilizing AcNPV and Sf9 cells has shown great potential for the synthesis of a variety of proteins [10]. Correct processing of exported proteins as well as post-translational modifications, have been reported to take place in this system [11,12]. The levels of expression (0.5–800 mg/l) are much higher than other expression systems making it an attractive alternative.

We report here on the expression of the α subunit of hCG in insect cells infected with a recombinant baculovirus carrying the *αhCG*, and characterization of the recombinant subunit in terms of its immunological and biological properties. We also present evidence that the bulk of the αhCG synthesized by the insect cells is not secreted into the medium, perhaps due to a 'secretory load' on the system. Finally, the implications of using a very late promoter for the synthesis of an extensively modified protein using baculovirus expression vector system are discussed.

Abbreviations: AcNPV, *Autographa californica* nuclear polyhedrosis virus; BEVS, baculovirus expression vector system; hCG, human chorionic gonadotropin; *αhCG*, gene coding for αhCG; h, hour(s); moi, multiplicity of infection; pfu, plaque forming unit; pi, post-infection; Sf9, *Spodoptera frugiperda* clone-9 insect cells; wt, wild-type

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2. MATERIALS AND METHODS

2.1 *Virus and cells*

The recombinant baculovirus – vAc*αhCG* – was constructed, characterized and plaque-purified as described [13]. AcNPV and

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vAc*αhCG* virus stocks were propagated in Sf9 cells growing in medium supplemented with 10% fetal calf serum [14].

2.2. *Detection and characterisation of human chorionic gonadotropin*

Culture supernatant was analysed for the presence of α subunit of hCG by radioimmunoassay [13,15], using a monoclonal antibody specific to the α subunit of hCG [16]. The biological activity of αhCG produced by Sf9 cells was determined [13,15] by employing the rat testicular receptor binding assay [17] and a mouse Leydig cell bioassay [18]. Briefly, an antibody dilution giving 30-40% binding of ^{125}I-hCG (40-60 μCi/μg, 10 000 cpm) in the absence of competing hormone was incubated with 0, 1.56, 3.125, 6.25, 12.5, 25, 50 or 100 ng/ml of αhCG standard (or culture supernatant dilutions), 40% normal horse serum and tracer. Incubations were carried out for 16 h at 4°C and immune complexes were precipitated using 66.6% v/v double-distilled alcohol and 6.6% w/v ammonium acetate. The biological activity of αhCG produced by Sf9 cells was determined by employing a rat testicular receptor binding assay and mouse Leydig cell bioassay. Medium containing 550 ng of αhCG was incubated with 12 μg of standard βhCG (prepared from commercial hCG of biological activity 10 000 IU/mg) for 16 h at 27°C. Formation of the $\alpha\beta$ dimer was analysed by a rat testicular homogenate radioreceptor assay. Rat testicular homogenate was prepared by a modification of an earlier method. Briefly, testes from adult Wistar rats were decapsulated and homogenized in 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM $MgCl_2$, 0.1% BSA and 0.1% sodium azide. The homogenate was centrifuged at 500 × *g* at 4°C and pellet resuspended in 8 ml of Tris buffer per pair of testes. 50 μl of the homogenate was incubated with 100 μl of different amounts of standard hCG or medium containing the dimer and 50 μl of ^{125}I-hCG (100 000 cpm, 40-60 μCi/μg) at 37°C for 2 h. The assay was terminated by the addition of 1 ml of cold Tris buffer. After centrifugation (2000 × *g*) the pellet was washed once in Tris buffer, and counted in a γ-counter. Dimer concentration was calculated by comparison with the inhibitory response of standard hCG. The capacity of the dimer to stimulate testosterone production was analysed by a mouse Leydig cell bioassay.

Adult NMRI male mice were sacrificed by ether inhalation, their testes were removed and minced in cold DMEM containing 0.38% $NaHCO_3$ + 2% FCS. The homogenate was gently stirred on a magnetic stirrer to help liberate individual cells. The cell suspension was passed through a nylon mesh and incubated at 34°C for 1 h under a 95% air + 5% CO_2 atmosphere. Cells were then centrifuged at 400 × *g* for 10 min at 4°C. The supernatant was discarded and 10 ml of fresh medium was added per pair of testes. 200 μl of this cell suspension was added into tubes containing 0, 1.56, 3.12, 6.25, 12.5, 25, 50 and 100 pg hCG or dilution of medium containing the dimer. Incubations were carried out for 3 h under conditions described above. Cells were killed by heating the tubes at 70°C for 15 min. RIA was carried out for testosterone, using WHO matched reagents as described in their Methods Manual.

2.3. *Protein analysis*

Sf9 cells seeded in a 24-well plate were infected with vAcαhCG virus at moi > 10. Total protein of infected cells were labeled with [^{35}S]methionine [14] and analyzed by PAGE.

3. RESULTS AND DISCUSSION

3.1. *Expression and biological activity of recombinant* αhCG

A partial restriction map of the recombinant vAc*αhCG* baculovirus [13] where the viral polyhedrin gene was replaced wth the *αhCG* cDNA is given in Fig. 1. In vAc*αhCG*, *hCG* was under the transcriptional control of viral polyhedrin gene promoter. The virus was plaque-purified and used to infect Sf9 cells seeded

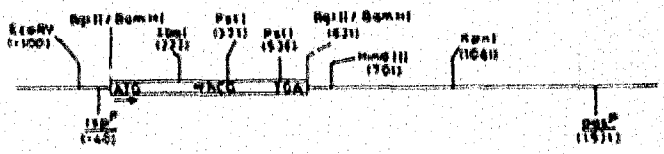

Fig. 1. Partial physical map of the site of homologous recombination in vAc*αhCG* showing restriction sites in *αhCG*. The transcription start point and the polyadenylation site of the viral polyhedrin gene is denoted as *tsp*^{p} and *pas*^{p}, respectively. The initiating methionine of *αhCG* is at + 149 and TAA is at + 423 with respect to *Bam*HI cloning site which was arbitrarily designated as + 1.

in a 24-well plate and at different times pi the medium was assayed for the presence of α subunit of hCG. Almost zero background values were observed in the culture media from uninfected or wt AcNPV infected cells. In contrast, α subunit of hCG was present in the medium of cells infected with vAc*αhCG*. The time-course of hCG secreted in the medium of vAc*αhCG*-infected cells is shown in Fig. 2. The levels of αhCG increased with time and reached a maximum of 11.3 μg/2 × 10^6 cells/ml at 72 h pi – an observation typical for genes under the transcriptional control of the very late polyhedrin gene promoter [10]. α subunit of hCG has been previously expressed in mammalian cells [7,8,15,19]. The level of expression in insect cells using baculovirus vector system, however, was significantly higher by more than an order of magnitude. This could be further increased using new improved baculovirus vectors.

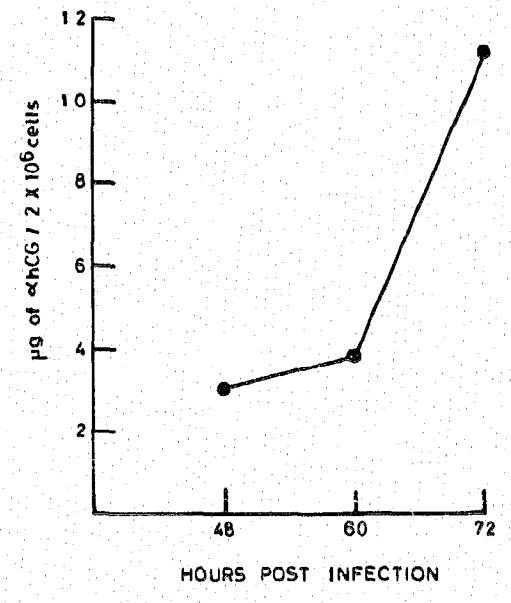

Fig. 2. Time-course of αhCG synthesis in insect cells infected with vAc*αhCG*. Cells were infected with vAc*αhCG* at an moi > 0.5 and *αhCG* expression was monitored at 48, 60 and 72 h pi, by a subunit specific radioimmunoassay. These results were the average of at least 5 experiments and variations in expression levels between experiments were never more than 0.1 μg.

The α subunit of hCG is not bioactive on its own, and requires an association with the β subunit to form a dimeric hCG which possesses biological activity [8]. Sf9 cells were infected with vAcα*hCG* and culture media were incubated with standard βhCG and the extent of $\alpha\beta$ dimer formation was estimated by a rat testicular radioreceptor assay and a mouse Leydig cell bioassay. Mock-infected Sf9 cells (Fig. 3) or those infected with wild-type virus did not exhibit biological activity either alone or in combination with externally added purified βhCG standard, in a mouse Leydig cell assay or receptor binding assay. Although αhCG was synthesized in vAcα*hCG*-infected cells (Fig. 2), these, however, did not exhibit bioactivity, unless annealed in vitro to purified βhCG. Both assays, therefore, clearly demonstrate the presence of biologically active αhCG in infected cell supernatant, which could associate with standard βhCG to generate the hormonal activity. Since the amount of dimer measured by the two assays was approximately the same, it implied that all the associated material had an agonistic characteristic.

3.2. *Immunoreactivity of Sf9-synthesized* αhCG

hCG contains about 50% carbohydrate in weight, and ~8.8% of the total protein weight is sialic acid [8,20]. Deglycosylated hCG binds to gonadal receptors with equal or higher affinity but the interaction fails to induce steroidogenesis [8]. Sialic acid apparently provides stability to the hormone circulating in the plasma. Desialylation, however, enhances its binding affinity for hCG receptors and also changes hCG from a full agonist to a partial agonist in testis and from a non-agonist to an antagonist in human thyroid [21]. αhCG synthesised in Sf9 cells infected with vAcα*hCG* is not only immunoreactive (Fig. 4) but shows a similar electrophoretic mobility as the authentic αhCG purified from urine of pregnant women. The α*hCG* gene has an open reading frame of 351 nucleotides and codes for a precursor peptide of 116 amino acids which, after cleavage of the 24 amino acid long signal peptide and extensive glycosylation, yields a mature ~21 kDa αhCG molecule as detected by SDS-PAGE. The absence of any detectable molecular weight difference between the authentic urinary and Sf9-synthesized αhCG, is in contrast to the synthesis of αhCG in mouse C127 cells [8] where it had a higher mobility than the standard α subunit. The observations that Sf9-synthesized αhCG and native αhCG are identical in terms of bioactivity and electrophoretic mobility, strongly suggest that the hormone made in this system is glycosylated such that there is no impairment of function. This is significant in view of the reported difference in the quality of glycosylation between insect cells and other eucaryotic cells [10]. It is also pertinent to mention that while insect cells are 'believed' to lack sialyl transferase – the enzyme that catalyzes the addition of sialic acid during protein processing – this deficiency, very interestingly, had no effect on the bioactivity of Sf9-synthesized αhCG. Thus, the expressed protein is authentic on the basis of (i) electrophoretic mobility (ii) immunoreactivity and (iii) biological activity.

3.3. *Secretion of* α*hCG in insect cells*

Insect cells and caterpillars, the host used for BEVS [22,23], can perform many of the post-translational modifications such as glycosylation, secretion, accurate peptide cleavage, phosphorylation and proper folding [10]. Foreign proteins which do not enter the secretory

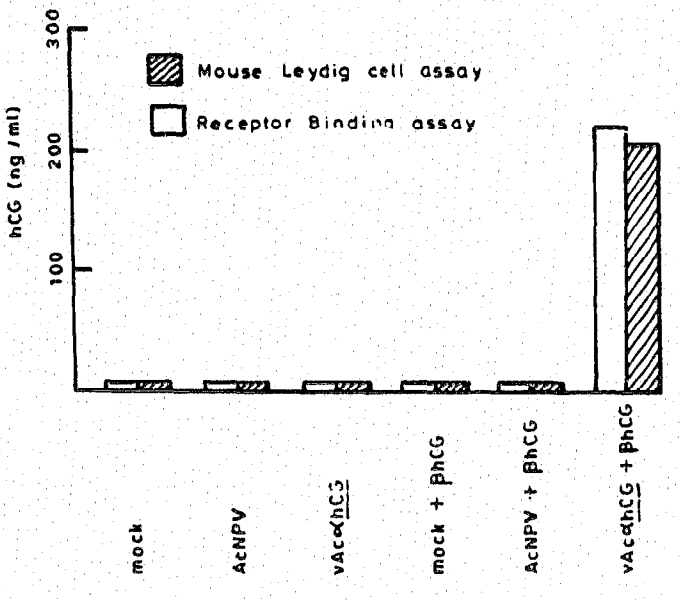

Fig. 3. Biological activity of expressed αhCG. Subunits of hCG are known not to interact with the receptor to any extent. Culture supernatant were incubated with a large molar excess of purified standard βhCG fro 16 h at 27°C and the αhCG/βhCG dimer was quantitated using a radioreceptor assay and a Leydig cell bioassay as described. Results presented herein were the average of at least 5 experiments and variations between experiments were never more than 10 ng.

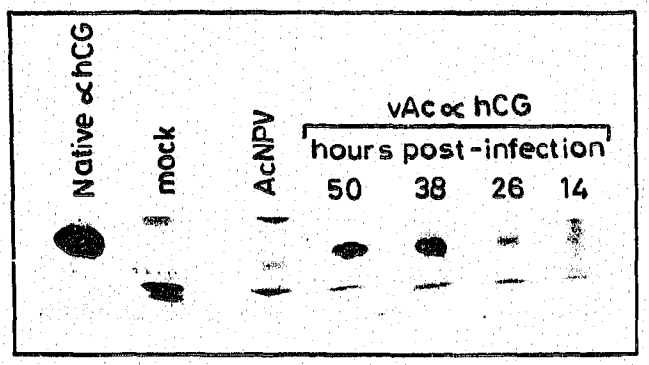

Fig. 4. Western blot analysis of total protein in infected cells. Total protein isolated from infected cells at 14, 26, 38 and 50 h pi were fractionated on 12.5% PAGE, transferred to nitrocellulose membrane and probed with monoclonal anti-αhCG antibodies. The synthesis of hCG is temporarily regulated, once again indicating that its expression is under the control of polyhedrin promoter.

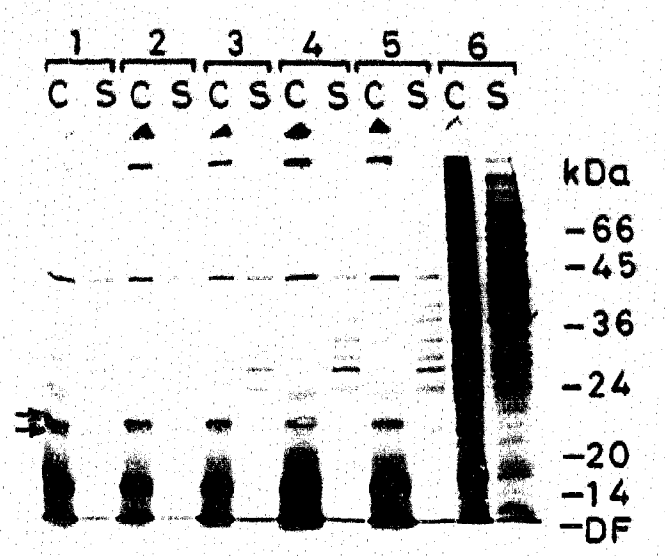

Fig. 5. [^{35}S]Methionine labeling of total proteins from virus-infected cells. Sf9 cells were infected with AcNPV (lane 6) and vAc*αhCG* (lanes 1–5) viruses and cells were labeled with [^{35}S]methionine as described [14]. Cells were pelleted, protein isolated from supernatant as well as cell pellet (after lysis) and fractionated on a 12.5% SDS-PAGE. Lanes 1–5 are replicates representing cell pellet (C) and supernatant (S) of vAc*αhCG*-infected cells. Arrows on the left indicate the positions of the non-secreted and secreted αhCG proteins. Protein molecular size marker is shown on the right in kDa.

pathway are synthesized to high levels (often close to the level of polyhedrin), but secretory and membrane-bound glycoproteins are synthesized to levels which are at least an order (or more) of magnitude less [12,24]. Polyhedrin, it should be mentioned, does not enter the secretory pathway. The over-production in these cells of a foreign protein, which has to be extensively processed (such as αhCG), perhaps puts a 'secretory load' on the cells. This could well be an explanation for the fact that the secretory pathway of these cells is compromised during the later stages of virus infection [12]. [^{35}S]Methionine labeling of total proteins synthesized by vAc*αhCG*-infected cells clearly demonstrated the presence of αhCG in the supernatant as well as in the cell pellet, though of a different molecular size. Those secreted into the medium moved with a slightly increased mobility (Fig. 5), due to the cleavage of the 24 amino acid secretory sequence. A significant portion of the αhCG synthesized by Sf9 cells was retained intracellularly. Video-densitometric scan of [^{35}S]methionine-labeled vAc*αhCG*-infected cell proteins revealed that ~61–72% of the αhCG synthesized by these cells is staying behind in the cells which did immunoreact to anti-αhCG antibody in a Western blot. Furthermore, we could also detect a ~12 kDa protein, present only in infected cells, corresponding to the size of the unprocessed αhCG which did not react with monoclonal or even polyclonal anti-αhCG antibodies in Western blot analysis. It is likely, that this also represents the overexpressed, unprocessed αhCG; unmodified due to cell death caused by the lytic nature of the baculovirus as has also been shown for tissue plasminogen activator [24]. One can then argue, that by giving more time to the cells, either by using a promoter activated earlier in the viral life cycle [24,25], in a virus-free system or by selectively silencing the lytic function(s) of the virus, it should be possible to avoid the 'secretory load'. These observations have implications in the use of conventional baculovirus vectors, where the transcription of a foreign gene is driven by the very late polyhedrin gene promoter, for synthesis of those foreign proteins which undergo extensive post-translational modifications. We are in the process of investigating the phenomenon of 'secretory load' on the system by expressing hCG under the control of a promoter [25] which is activated earlier than polyhedrin and is not as strong.

Acknowledgements: This work was supported by a grant to S.E.H. (no. BT/TF/3/26/7/88) from the Department of Biotechnology, Govt. of India.

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